Immediate and long-term effects of testosterone on song plasticity and learning in juvenile song sparrows

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ABSTRACT

Steroid sex hormones play critical roles in the development of brain regions used for vocal learning. It has been suggested that puberty-induced increases in circulating testosterone (T) levels crystallize a bird's repertoire and inhibit future song learning. Previous studies show that early administration of T crystallizes song repertoires but have not addressed whether new songs can be learned after this premature crystallization. We brought 8 juvenile song sparrows (*Melospiza melodia*) into the laboratory in the late summer and implanted half of them with subcutaneous T pellets for a two week period in October. Birds treated with T tripled their singing rates and crystallized normal songs in 2 weeks. After T removal, subjects were tutored by 4 new adults. Birds previously treated with T tended toward learning fewer new songs post T, consistent with the hypothesis that T helps to close the song learning phase. However, one T-treated bird proceeded to learn several new songs in the spring, despite singing perfectly crystallized songs in the fall. His small crystallized fall repertoire and initial lag behind other subjects in song development suggest that this individual may have had limited early song learning experience. We conclude that an exposure to testosterone sufficient for crystallization of a normal song repertoire does not necessarily prevent future song learning and suggest that early social experiences might override the effects of hormones in closing song learning.

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1. Introduction

Steroid hormones have important effects on memory formation, including vocal learning, in birds (Saldanha et al., 1999). Both estrogens and androgens have organizational effects on development of the song control system and on song learning behaviors (Gurney and Konishi, 1980; Bottjer and Johnson, 1997; Schlinger, 1997; Fusani and Gahr, 2006). Estrogens may act to promote learning and memorization of songs and may be required for maintaining the brain plasticity necessary for keeping the sensitive phase open (Bottjer and Johnson, 1997; Schlinger, 1997). In contrast, androgens appear to function to promote crystallization of song production (motor stereotypy), and may terminate the song memorization phase (Bottjer and Johnson, 1997; Schlinger, 1997). Each of the major song control nuclei contains androgen receptors, and HVC also contains estrogen receptors (Schlinger and Brenowitz, 2009).

Experiments examining the role of androgens in song crystallization and song learning have been carried out on four species of Emberizine sparrows, song sparrows (*Melospiza melodia*), swamp sparrows (*M. georgiana*), white-crowned sparrows (*Zonotricia leucophyrs*) and dark-eyed juncos (*Junco hyemalis*). In all of these species song learning is completed in the first year – they are 'closed-ended' learners (Beecher and Brenowitz, 2005) – and all or most song memorization occurs in the bird's natal summer (the 'critical period' or 'sensitive phase'). Marler et al. (1988) castrated young song sparrows and swamp sparrows and found that these birds failed to crystallize their songs within the first year as they normally would. Application of testosterone (T) when the birds were more than a year old induced rapid song crystallization and when T implants were removed, the birds regressed to plastic song. Whaling et al. (1995) tape-tutored juvenile white-crowned sparrows during their sensitive period (days 10–100) and treated them with T implants between days 100 and 130, i.e., between the end of the song memorization phase and the beginning of the production phase. T-treated birds crystallized their songs within two weeks, or approximately 5 months earlier than control birds. However, the songs of the T-treated birds were abnormal, resembling those of isolate birds. They were abnormal not only during the early

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T-induced crystallization, but again the following spring, long after T implants had been removed. In contrast, dark-eyed juncos developed structurally normal song in two studies with very different schedules of T administration. Titus et al. (1997) captured juvenile juncos in the field in October and November, long after the sensitive period, and administered T to them in late January during the early part of the motor phase when birds were singing plastic song. Song structure in T-treated birds was normal although these birds had smaller song repertoires and sang less than control birds. Gulledge and Deviche (1998) captured juvenile juncos in the field in mid-September, and administered T implants shortly thereafter, in the silent gap between the end of the memorization phase and beginning of the motor (production) phase. Birds began to sing two weeks after T implants and song structure appeared normal. Finally, one study with zebra finches (*Taeniopygia guttata*), a species in which the memorization phase of song learning is short and is overlapped by a relatively short production phase, found that early T administration induces premature crystallization of incompletely developed song (Korsia and Bottjer, 1991).

The one clear conclusion emerging from these studies is that T administration either ahead of schedule in normal birds, or later in life in castrated birds, produces song crystallization. On the other hand, these studies provide conflicting answers to the question of whether early song crystallization induced by T administration will lead to normal song development: under these conditions, white-crowned sparrows and zebra finches developed abnormal song whereas juncos developed normal song. It is possible that song development proceeded more normally in the junco studies because song memorization had occurred in the field, whereas in the white-crowned sparrow song memorization occurred in the lab with tape-recorded song. There is considerable evidence that birds are more apt to learn from live song tutors than from tape tutors (reviewed in Beecher and Brenowitz, 2005) and in fact, some of the strongest evidence for this comes from these species (Beecher, 2008). A second possible explanation for this difference relates to variation in the time period when T was administered. Zebra finches are the only species in which T was administered during the early sensitive period (among other time periods) and it is clear that the timing of T administration has dramatic effects on crystallized repertoires in this species (Korsia and Bottjer, 1991).

Although these studies have made great strides in elucidating the relationship between T and song crystallization, none of the previous studies addressed the question of whether T-induced premature crystallization also prevents further song learning. Note that in closed-ended learners (all the species considered so far), normal crystallization marks not only the bird's arriving at its species-typical song repertoire, but finalizing that repertoire, i.e., no new songs will be added nor old songs subtracted in subsequent years. In the present study, we investigated the role of T in song crystallization and learning of new songs in juvenile song sparrows. Song sparrows are closed-ended song learners and males develop repertoires of 6–13 song types in the first year of life (Peters et al., 2000). Typically much of the song learning takes place during an early sensitive phase in the natal summer (Marler and Peters, 1987), although song sparrows can continue to learn new songs throughout the first year of life under some circumstances (Nordby et al., 2001; Nulty et al., 2010). In this study, we treated wild-caught juveniles with T for two weeks after the conclusion of the summer sensitive period and exposed them to new live song tutors after removing the implants. We asked five specific questions. First, will T administered to juveniles in the autumn cause birds to prematurely crystallize their song repertoires as predicted by previous work? Second, how quickly will T change song rate and song structure? This process takes several months in unmanipulated wild birds in our field population. Third, will the songs in these early-crystallized song repertoires have normal acoustic structure? Fourth, will these

early-crystallized song repertoires be of normal size? Fifth, will early transient T prevent subsequent learning of new songs? That is, will a dose of T sufficient to crystallize a bird's song repertoire also inhibit future song learning? On the basis of the studies reviewed above, we predicted that T would prematurely crystallize song repertoires and this would happen within two weeks. Because T would be administered after early song memorization and because that phase would be taking place under natural field conditions, we also predicted that crystallized songs would have normal acoustic structure. At the same time this early field experience might handicap our test of the fifth question, for once song sparrows have learned songs under normal field conditions, they are much less likely to learn songs in the lab (Beecher and Brenowitz, 2005; Beecher, 2008; Nulty et al., 2010). Nonetheless, we concluded that the benefit of using early field tutors would offset the disadvantages and predicted that if T acts to prevent future song learning, the majority of late song learning from lab tutors should be seen in the control subjects.

2. Methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington (Protocol Numbers 2207-03 and 2008-06). We released all subjects at the site of capture following the experiments. Since subjects had substantial field experience as juveniles we felt that they might have good chances of survival and we confirmed that at least one subject later obtained a breeding territory at the field site.

2.2. Subjects

We brought eight juvenile birds into the laboratory from our field site (Discovery Park, Seattle, WA) between 28 July and 2 August 2005. All birds were independent of their parents when collected. We estimated the birds to be between 50 and 100 days old. These juveniles had been exposed to song in the field for most or all of the classical sensitive period for song memorization (primarily 20–60 days: Marler and Peters, 1987). Birds were identified as juveniles by their distinctive brown streaky juvenile plumage (Pyle, 1997) and/or their production of subsong. We collected only juveniles with wing chords $\geq$66 mm, to be sure that we had only male subjects, a relatively good measure for song sparrows in our population and in general (Nice, 1937). The sex of each bird was confirmed through genetic testing using PCR sex analysis on a small drop of blood ("Sex Made Easy," Zoogen Inc.; www.zoogen.biz).

In the laboratory each bird was placed in a cage (dimensions: 40 cm × 23 cm × 26 cm), and given ad lib. food and water. The cage was placed in a sound attenuating chamber (for details see Beecher et al., 2007) so that each bird was isolated from the vocalizations of all other birds. Each chamber was equipped with two loudspeakers (one on either end of the chamber) and a microphone connected to a PC computer. The computer was programmed to record and save all songs produced by each subject throughout the experiment using the sound detector module in Syrinx (www.syrinxpc.com; John Burt). Because of hardware limitations, only four subjects could be recorded on a given day, so individual birds were recorded every other day. For analyses, we pooled birds sampled in both days. Birds were housed under a natural Seattle photoperiod, which was controlled by an astronomical timer. All subjects were released at the capture site upon completion of the experiment.

Table 1
Song stages used for scoring song development.

Score	Stage	Song description
NA	No song	Not singing.
1	Early subsong	Quiet, unstructured, non-repeating sounds. Not resembling adult song sparrow syllables.
2	Late subsong	Still fairly unstructured, but there are some elements that start to resemble song sparrow song. Lacking trills and buzzes, with other song elements poorly developed.
3	Early plastic	Recognizable song sparrow elements (still somewhat wobbly), including trills or buzzes. Elements not organized into distinct songs.
4	Late plastic	These are "attempts at songs," in terms of structure and syntax. Individual syllables are better developed and are somewhat repeatable. Long trains or combined songs still occur.
5	Crystallized	Adult song. All notes are well formed and repeatable. Songs produced in discrete form, not in multi-song combinations.

2.3. Testosterone implants

After two months in isolation, half of the subjects received a subcutaneous implant of T on 8 October. Implants were made from Silastic tubing (i.d: 1.0 mm; o.d: 2.0 mm; length: 12 mm; VWR, West Chester, PA) filled with crystalline T, as in Tramontin et al. (2003). The implants were rinsed with ethanol and soaked overnight in 0.1 M phosphate-buffered saline (PBS) prior to subcutaneous implantation over the pectoral muscle. Prior to implanting the subjects, we carefully reviewed several days' worth of songs for all birds to determine their overall song development (see below). Each bird was ranked relative to the other birds by his song development score, and T and control treatments were assigned to pairs of birds based on these ranks, i.e., one of the two most developed birds was randomly chosen to receive the treatment while the other became a control subject, and so on down to the least developed pair of birds. Control birds received an empty Silastic capsule. Implants were removed after two weeks on 20 October. Although we do not know specific ages for any of the birds, we expect that birds were implanted on 100–160 days, similar to the manipulation in white-crowned sparrows by Whaling et al. (1995).

2.4. Assessment of song development

To assess song development, we used a 5 stage song development scoring system (Table 1). Each song was ranked from least to most developed, and categorized as follows: (1) early subsong, (2) late subsong, (3) early plastic song, (4) late plastic song, and (5) crystallized song (see Table 1 for detailed definitions). Though song development presumably is a graded process, these delineations are based on conventionally described phases of song learning (Catchpole and Slater, 2008) and provide a good measure of song progression for our population of song sparrows. We found that our scoring was highly repeatable both within and between observers (repeated scoring between observers produced identical scores for ∼70% of songs and scores within ± one developmental stage for 100% of songs scored). For each bird, we scored the first 100 songs he sang each day, starting a week prior to the T implants (30 September) and continuing every other day until the day T implants were removed (20 October).

As a second measure of song development, we took the number of distinct song types we detected in a bird's song repertoire at the end of the T treatment. Song sparrow repertoires range from 5 to 13 song types, but most adults in our population sing repertoires of 7–13 song types (Beecher et al. unpublished data). On the low end, 6-song repertoires are uncommon (<5% of cases) and 5-song repertoires are rare (∼1% of cases). Thus the final repertoire size of the experimental birds before implant removal could also serve as a measure of song development. This variable has not been measured in the previous studies of hormone manipulation discussed above, probably because two of the species have one-song repertoires (white-crowned sparrows and zebra finches) while the other two species (juncos, swamp sparrows) have very small repertoires.

In addition to song development, we measured singing rates for each subject on the same days (30 September–20 October). We used the number of songs detected by Syrinx to count the total number of songs each bird sang on each sampling day. We counted each utterance followed by silence as a song, so subsong and plastic song length tended to be slightly longer than crystallized song lengths (Table 1). Measurements of the number of songs per day were verified for each subject by periodically scanning the sound file clips of songs detected by Syrinx. The detector rarely produced false detections.

2.5. Tutoring regime

Subjects were tutored in the autumn (22 October–20 November; beginning shortly after the prebasic molt) and in the spring (22 December to 1 April), following singing patterns typical for our population. In our population, wild song sparrows sing very little in late fall and early winter but increase singing rates and territorial behavior rapidly with the lengthening days following the winter solstice (Smith et al., 1997).

We used four live adult tutors in this study. Adult tutors were collected from a different population (in Eastern Washington) than juvenile subjects and therefore sang songs that were quite different from those that the subjects had previously heard in the field. The four tutors were separated into two pairs of "neighbors," who were permanently located in two different rooms (i.e. tutors RY90 & RY94 in one room; tutors BOO & BG in another). Tutors were housed in their normal cages (dimensions: 45 cm × 28 cm × 18 cm), which were located approximately 1 m apart in visual and acoustic contact. Each juvenile was individually exposed to a pair of tutors by placing his cage approximately 1 m from each of the adult's cages, so that the three cages formed points of a triangle. This design allowed the young bird to both interact vocally with the potential tutors and to eavesdrop on the song interactions between the two tutors, a powerful stimulus for song learning in this species (Beecher et al., 2007; Templeton et al., 2009). Tutor song rates varied by day, but generally ranged from 50 to 700 songs per tutor per day. Subjects were rotated through the rooms on an 8 day schedule, so that each juvenile received an entire day's tutoring in one room, followed by 3 days in isolation, followed by tutoring in the other room, followed by 3 days of isolation, etc. Unlike the pre-tutoring isolation, however, juveniles continued to hear songs while isolated during this phase of the experiment. We used a "virtual tutor" computer program written in MatLab (see Burt et al., 2007 for details) to simulate the interactions of the adult tutors (tutors interacted with each other, but not with the subject). Juveniles heard the same tutors (500 songs per tutor per day) in their isolation chambers to which they had previously been exposed, i.e., after being in the room with BOO and BG, subjects heard BOO and BG in their isolation chambers for three days before being moved to the room with RY94 and RY90, after which they would hear these two birds in their isolation chamber. Juveniles were rotated between rooms at noon each day, so that each subject received uninterrupted tutoring sessions during the times when the adults had the highest singing rates (morning and evening). Thus, during the four weeks immediately after the T

implants were removed (22 October–20 November), subjects were tutored by live birds on a total of 7 days and by recordings of these birds on 21 days. The same tutoring procedure was followed in the spring tutoring (22 December–1 April).

2.6. Analysis of song learning

To determine how many songs each bird learned from the laboratory tutors, we followed the conventional procedure for birds like song sparrows that have repertoires of complex songs (e.g., Marler and Peters, 1988; Marler et al., 1988; Peters et al., 1992; Nowicki et al., 1999; Beecher et al., 2007): the similarity of subject songs to potential tutor songs was judged visually, using sonograms of the songs, by several independent judges. We printed large sonograms that included all variations (Stoddard et al., 1988; Podos et al., 1992) of each subject's and each lab tutor's songs for visual comparison. Scoring was done blindly, i.e., without knowledge of a bird's experimental treatment. We assigned a tutoring score for each of the subject's songs. A tutoring score was based on the song's similarity to the songs of each lab tutor and ranged from 0 to 4, where each point corresponded to a segment of the song shared by tutor and subject. Thus, 0 = no match, 1 = one shared segment, 2 = two shared segments, 3 = very good match, with only slight differences, 4 = nearly identical songs. Because song sparrow songs can often be broken into roughly four segments, a score of 2 would represent an approximate 50% match. Songs were credited to a lab or field tutor in an all-or-none fashion: if the match to a lab tutor song was scored at 2 or above, it was credited to the lab tutor, otherwise it was credited to the (unknown) field tutor. We then calculated the proportion of each subject's repertoire that was learned from the new, post-treatment laboratory tutors. We used this method because it was the most conservative with respective to our original hypothesis (that T prevented further song learning). To be sure that we did not overestimate learning, we also recalculated post-T learning scores based on the percent of all phrases (i.e. 1–4 points per song type) learned in the laboratory; these results did not differ from the previous analysis so we report only the first analysis in the results section.

Because for the T-treated birds, we had fall crystallized repertoires as well as their final spring repertories, we were able to check the validity of our method of assigning lab tutors. We first analyzed the bird's final (spring) crystallized repertoire and assigned lab tutors as just described. We then reanalyzed this repertoire using the bird's early, fall crystallized repertoire as a "fifth tutor." This "fifth tutor" served as a proxy for songs that had been learned in the field in the summer. Any difference in tutoring scores between these two analyses would represent "false positives" (i.e., cases of attributing a song as learned from a laboratory tutor when it had actually been learned in the field). There were no false positives of scores 2 or above. The only misattributions to lab tutors had received scores of 1, and so, as noted above, were not counted. We believe that the absence of false positives is due to the tutors having been collected from a different population than were the juvenile subjects, and therefore having songs that were quite different from those songs the subjects had previously heard in the field. To calculate the proportion of each subject's repertoire that was learned from the new, post-treatment laboratory tutors, we divided the number of his songs credited to lab tutors by the subject's total repertoire size.

2.7. Statistical analyses

We used a General Linear Mixed Model to assess changes in song rate and song development, with treatment, date, and the treatment-date interaction as factors. Subject ID was used as a random factor to control for variation among individuals. Data were
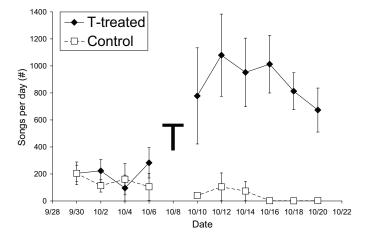

Fig. 1. Testosterone causes an increase in singing behavior. Mean daily song rates ($\pm$ standard error) are shown for T-treated and control subjects across time. T implants were administered on 10/8 so the birds were not sampled on this day.

not transformed. Because we found overall effects, we also ran post hoc t-tests by specific dates to determine at exactly which time point the two treatment groups diverged relative to implant of the T pellet. We also compared the post-treatment song learning scores of T-treated and control birds using a t-test. All statistics were calculated in JMP 7.0 and are based on two-tailed tests.

3. Results

3.1. Song rate

T implants increased song rates (Fig. 1). The model showed effects of treatment (T implant vs. control, $F = 13.2$; $P < 0.001$), the date relative to T treatment ($F = 2.73$; $P = 0.04$), and the interaction between treatment and date ($F = 3.49$; $P < 0.01$). There were no differences in singing rates between control and treatment birds prior to T implants (t-tests, $P > 0.60$), with birds in both groups singing an average of approximately 200 songs per day. T-implanted birds increased singing rates to more than three times the rate of control birds within two days (10 October: $P = 0.08$), with the two groups diverging further subsequently (12–20 October: all $P < 0.01$). While treatment birds rapidly increased singing rates, inspection of Fig. 1 suggests that control birds' singing rates declined over time, as is typical of autumn singing behavior in wild song sparrows.

3.2. Song development

T implants maintained singing in the experimental birds (Fig. 1), and led to crystallization of their songs (Fig. 2). We found overall effects of treatment ($F = 7.5$; $P = 0.004$), date ($F = 59.4$; $P < 0.0001$), and an interaction between treatment and date ($F = 35.5$; $P < 0.0001$) in the model. We saw no difference in song development between groups before T treatment ($P > 0.3$ on all days), with all birds receiving scores indicative of late subsong (scores of ~2). However, within two days (the first sampling period), T-treated birds sang more full-developed songs than control birds, with their average song scores increasing by an average of 1.6 points. On this date and all subsequent dates, T-treated birds produced more highly developed songs than control birds (t tests, $P < 0.01$ for all). Song development continued rapidly over the next two days, with bird's scores improving an additional 1/2 point, on average, and then beginning to level out somewhat. By 20 October (two weeks after T implants), all T-treated birds were producing noticeably more developed songs, with almost all of the songs produced classified as 'crystallized' and an average
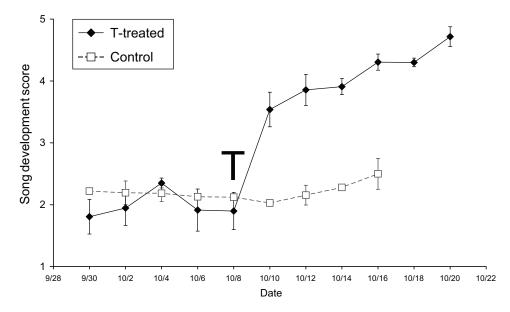
Fig. 2. Testosterone implants lead to rapid development of song structure. Means (± standard errors) are shown for T-treated and control birds across time. T pellets were implanted on 10/8; song development levels for this date are based on songs produced prior to implanting the birds.

song score of 4.7 ± 0.16 (two birds sang only crystallized songs, two sang late plastic songs in addition to crystallized songs). Although birds generally stopped singing after the T implants were removed and we do not have data on the stereotypy of their songs immediately post-T, our recordings from early spring 2006 indicate that all birds reverted to plastic song at some point after T implant.

Fall song repertoires appeared normal, with a mean repertoire size of 8.8 ± 1 songs. The repertoire sizes of the four experimental birds varied substantially among individuals. Post-T fall repertoire sizes and stereotypy scores for these birds are shown in Table 2 (control birds still sang undeveloped sub/plastic song at this stage, so it was not possible to assess repertoire size). Most T-treated birds developed normal sized repertoires, but one experimental bird, CRRM, developed only 5 song types (and two of these types contained several shared elements). This bird's songs were well developed (average song stereotypy score of 5/5 on the last day of T), but his full repertoire was unusually small.

3.3. Post-treatment song learning

All birds crystallized normal song repertoires the following spring, with an average repertoire size of 10 ± 0.7 song types. The overall rate of de novo learning of laboratory tutor songs was relatively low, with control birds learning around 1/3 of their songs in the laboratory (overall mean = 32% ± 16; range = 0–64%, with 3 of 4 birds exhibiting some clear de novo learning). There was a trend for T-treated birds to have lower rates of de novo song learning, with only 1 of 4 birds learning new songs after they were implanted (overall mean = 9% ± 9; range = 0–38% of repertoire learned in the

lab). However, this difference was not statistically significant (t-test: $t = 1.21$, $P = 0.2$; Fig. 3). Surprisingly, the bird that learned new songs after early song crystallization due to the T implant actually learned more than a third of his final song repertoire de novo.
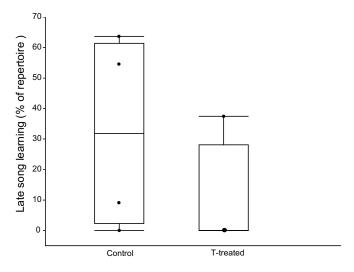

Fig. 3. Post treatment song learning for testosterone-treated and control birds. Three of four control subjects and one of four treated subjects learned new songs de novo. Control birds tended to incorporate more new songs into their repertoire than T-treated birds, though this difference was not statistically significant ($P = 0.2$). Box-plots show median, 25%, 75% quartile, minimum, and maximum values for subjects (points) in each treatment.

Table 2
Song development scores and repertoire sizes of T-implanted subjects across time. Scores are shown for time periods before implant ('Pre T'), after 2 weeks of T ('Post T'), and when subjects were adults with finalized song repertoires ('Final'; Spring–Summer 2006). The subject that learned de novo songs in the laboratory began at a low song developmental stage and crystallized a small repertoire during T treatment, despite singing perfectly stereotyped songs.

Subject	Pre T stereotypy score	Post T		Final repertoire size	Learned new songs post T?
		Repertoire size	Stereotypy score		
gim	2	9	4.7	11	No
wwam	2.5	11	5	11	No
bypm	2	9	4.4	10	No
crrm	1	5	5	8	Yes

4. Discussion

We found that early exposure to T crystallized song structure in young song sparrows, that T-induced crystallization was rapid and produced a repertoire of normal songs, and that crystallization the following spring was likewise normal. Research on the role of T in song learning has a rich history (Marler et al., 1988; Korsia and Bottjer, 1991; Whaling et al., 1995; Titus et al., 1997; Gulledge and Deviche, 1998) and our study adds to this literature by examining both prematurely crystallized repertoires (T-induced after the summer sensitive period) and subsequent crystallized repertoires occurring the following spring, after additional late song tutoring. Our detailed examination of song development after T implantation indicates that the hormone rapidly advances song development, with noticeable maturing of song structure and increased singing rates after only 2 days. Overall song crystallization took approximately 2 weeks for most birds treated with T, similar to the rates described for other related species after T implants (1–3 weeks for swamp sparrows and white-crowned sparrows, Marler et al., 1988; Whaling et al., 1995) but much shorter than observed naturally in the field (2–3 months minimum in our population).

We found that T not only affected song structure, but also produced a rapid and dramatic increase in song output, with birds tripling their rate of singing within 2 days of T treatment. In contrast, a study of juvenile juncos found that young birds actually decreased song output following T implants (Titus et al., 1997). One possibility is that T acts differently at different life stages; our experiment implanted birds in the autumn (early October), whereas Titus et al. implanted birds in the winter/spring (early January). Consistent with this possibility, Gulledge and Deviche (1998), also working with juncos, implanted birds in September and found that T generally produced higher singing rates.

Previous experiments that exposed young birds to premature high T levels reported reduced song quality in adults, with songs generally resembling those of birds lacking song tutoring altogether (Marler et al., 1988; Korsia and Bottjer, 1991; Whaling et al., 1995). In contrast, we found no evidence of reduced song quality in our experimental birds' adult song repertoires, which we suggest may have been due in part to the early song memorization occurring in the field rather than in the lab as in these earlier studies. However, because we did not manipulate this variable, any of the many other differences between the other experiments and ours could explain the different outcomes of these experiments.

In contrast to the early crystallization results, it is somewhat more difficult to interpret our findings on song learning following early T exposure. To begin with, although the difference between control and T-treated birds was relatively large – 9% vs. 32% de novo late learning – our statistical power was low due to the small sample size and this difference was not significant. Second, although the overall pattern was generally toward reduced song learning in the T-treated group of birds, one of the experimental birds learned a substantial portion of his final repertoire after initially crystallizing a different song repertoire in the fall. Although only one T-implanted bird demonstrated this pattern, our finding indicates that, at least under some circumstances, exposure to high levels of T is not sufficient to completely inhibit additional song learning, despite being sufficient to cause song crystallization. This one case takes on additional weight given the fact that late learning was uncommon even in the untreated birds (shown by 3 of the 4 controls, but only 32% of songs learned de novo overall). In other studies with juvenile song sparrows brought into the laboratory at or near the end of the classical sensitive period (roughly May, June and early July in our population) we have observed similar low rates of de novo song learning (Nulty et al., 2010, Beecher et al., unpublished observations). The late song tutors not only have the disadvantage of not appearing until after the primary song learning period is over, but also of functioning under the presumably less stimulating laboratory conditions. It would be interesting to repeat the present experiment with young birds hand-raised in the lab. Under these circumstances young sparrows are much more likely to learn songs from late tutors (Baptista and Petrinovich, 1984; Nordby et al., 2001). Finally, it is possible that had we continued the T-treatment somewhat longer, it might have taken the T-treated birds to a more complete – and irreversible – crystallization (Price, 1979; Lombardino and Nottebohm, 2000). Although two subjects still sang some late plastic songs in addition to crystallized songs at the time the T implants were removed, the other two subjects, including the one who went on to learn new songs, sang nothing but crystallized songs at the time when the T implants were removed. Thus, it appears that a dose of T sufficient to fully crystallize the songs a song sparrow sings does not necessarily prevent later addition of songs to the repertoire, at least for some individuals.

Variation among individuals in late learning may reflect variation in their developmental histories upon entering the lab. For example, it is possible that the one individual in the T-treated group (CRRM) who learned new songs in the lab after initially crystallizing his repertoire had heard and memorized fewer songs on entering the lab. This bird was captured at the same time as the other subjects but he had a rather low song development score (mid subsong) when he was first brought to the lab (August), though another bird (who learned very little, despite not receiving a T implant) had a similar song development level. Prior to T-treatment (October 20), he still had one of the lowest song development scores and he did not sing in the first post-treatment sampling period (2 days post T). After T administration, his song development initially lagged behind other birds, but after a week it was equivalent, and at the end of the implant period, he sang perfectly crystallized songs with the highest possible average daily score. Thus, the T clearly had a potent effect on his singing rate and song stereotypy, but still did not block additional song learning. Although he sang perfectly formed songs at the end of T-administration, his crystallized repertoire was rather small for a song sparrow, with just 5 song types (most birds have 7–12 types). This small repertoire and initial low song development score suggest that this individual may have had limited song learning experience in the wild; he may have heard and thus memorized only a small number of songs before T exposure. We know from previous work that reduced early song exposure can delay the close of the sensitive phase in birds (Kroodsma and Pickert, 1980; Jones et al., 1996), Kroodsma and Pickert (1980) showed that in marsh wrens (*Cistothorus palustris*), a repertoire species like song sparrows, young birds that do not hear enough songs in the summer (as might happen with late-hatching birds in the wild) will continue to add songs to their repertoire the following spring. His small fall repertoire (of well-formed songs) suggests that this may have been the case for our subject CRRM, and may explain why he learned new songs in the lab the following autumn/spring. Better understanding how early social experiences affect late song learning may be key for developing our understanding of the processes of vocal learning in animals Further experiments examining how social and hormonal factors interact to affect song crystallization and future learning are clearly needed.

Acknowledgements

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